REMARKS

Claims 24-46 have been examined. In this response, Claims 31 and 34 have been cancelled, and Claims 24, 32, 35, and 37-39 have been amended. None of these amendments introduces new matter: support for Claim 24 as amended is found in original Claim 31, and the remaining amendments correct claim dependencies. Claim 38 has also been amended to correct a typographical error. Favorable reconsideration and allowance of all pending claims are respectfully requested.

Applicants thank the Examiner for the courtesy of an interview on December 10, 2002, during which the enablement rejection was discussed and Applicants agreed to point out other enabled uses of the presently claimed subject matter. In addition, Kaplitt, M.G. *et al.* (1996) *Ann. Thorac. Surg.* **62**:1669-1676 (hereinafter "Kaplitt"), a reference already of record, was discussed. In view of Kaplitt, the pending claims have been amended to specify the amount of recombinant AAV vector infused into the coronary artery or coronary sinus.

The Enablement Rejection

Claims 24-46 have been rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to enable one skilled in the art to use the invention.

The present claims are directed to an infusion method to introduce a desired nucleic acid into cardiomyocytes using particular concentrations of recombinant adeno-associated virus (rAAV). The Examiner has acknowledged that such claims are enabled with respect to delivery of a marker gene. For example, Applicants demonstrated that rAAV encoding lacZ transduces 40-50% of cardiomyocytes when about 10^9 IU were delivered by infusion into a coronary artery of a mouse heart.

Hence, gene transfer is enabled. With the demonstration of a stable and efficient gene transfer method into cardiomyocytes, those of skill in the art at the time of the invention would have immediately recognized many specific and substantial, credible uses of the claimed method. Such uses include, but are not limited to, (1) delivery of marker genes to study gene expression in the heart; (2) delivery of therapeutic genes to treat acquired and inherited cardiac conditions, and (3) delivery of disease causing genes to create organ and animal models useful in developing therapeutic drugs for those specific diseases. With respect to studying gene expression, those of skill in the art would know that the marker genes could be coupled to promoters (or promoter fragments) and promoter activity assessed from the level of marker gene expression. Methods for measuring and evaluating gene expression have long been known in the art. For example, with the appropriate promoters controlling expression of a marker gene, the presently claimed method can be used to evaluate the regulation and expression of genes in the functioning heart, including hearts in particular disease states. In fact, one such study was done using rAAV to evaluate the cardiac muscle-specific alpha myosin heavy chain promoter [Aikawa *et al.* (2002) J. Biol. Chem. 277:18979-18985; hereafter "Aikawa"].

The law is clear that a single enabled use is sufficient to satisfy the requirements of 35 U.S.C. 112, first paragraph. *See Raytheon v. Roper*, 724 F.2d 951 (Fed. Cir. 1983); *In re Gottlieb*, 328 F.2d 1016 (C.C.P.A. 1964); MPEP §2107.02. In light of the above use relating to to evaluation of gene expression in cardiomyocytes, which would have been readily apparent and a well-established utility to those of skill in the art upon reading Applicants' specification, this enablement rejection should be withdrawn.

In addition, the record provides and Applicants have enabled other uses of the claimed method. For example, Applicants submitted the Parmacek Declaration, in which Dr. Parmacek

opined that those of skill in the art at the time of the invention would recognize further utilities for the claimed invention, including uses to deliver genes to establish organ models and animal models for human cardiovascular disease (Parmacek Declaration, ¶ 22). Another example of a readily-apparent, well-established use is to provide a marker gene as a control for experiments in which therapeutic genes are provided. For example, Kawada used rAAV encoding lacZ as a control for somatic gene therapy for dilated cardiomyopathy (DCM) in the TO-2 hamster strain receiving rAAV encoding the δ-sarcoglycan (δ-SG) gene (Kawada *et al.* (2002) Proc. Natl. Acad. Sci. USA 99:901-906; hereafter "Kawada").

Moreover, as already established on the record, the expressly-identified use of the claimed subject matter—cardiac gene therapy—is enabled without undue experimentation. However, to further bolster this argument, the Kawada reference demonstrates delivery of a therapeutically-effective amount of a gene product to treat a cardiac condition using Applicants claimed methods. Kawada states "that, upon rAAV-mediated efficient δ-SG gene transfer into the heart, TO-2 hamsters can be rescued from developing DCM and survive for at least 40 weeks, which exceeded the lifespan of TO-2 heart without responsible gene transductions, thus drastically improving the disease prognosis" (p.904, right column, first sentence of Discussion). While those experiments were conducted via intramyocardial injection (see, *e.g.*, Figs. 5 and 6), Kawada indicated expression was obtained using intracoronary delivery ("Other methods of gene transfer *in vivo* using intracoronary administration and/or electroporation did not exceed the present level of intramural administration (. . ., unpublished data)"). Kawada thus represents post-filing evidence that Applicants' invention works as claimed.

Finally, there is no need for Applicant to demonstrate that the present methods provide "clinical efficacy" to establish enablement for the presently claimed invention. *See In re Isaacs*,

347 F.2d 889 (CCPA 1963); *Ex parte Balzarini*, 21 USPQ2d 1892 (Bd. Pat. App. and Int. 1991); *In re Brana*, 51 F.2d 1560 (Fed. Cir. 1993).

In view of the foregoing arguments, and the extant record, Applicants believe the presently claimed subject matter is enabled and respectfully request withdrawal of this rejection.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit the present claims are in condition for allowance, which action is earnestly solicited. The Examiner is invited to contact the undersigned by telephone should any issues remain outstanding.

Respectfully submitted,

March 24, 2003

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Cardiomyocyte-specific Gene Expression Following Recombinant Adeno-associated Viral Vector Transduction*

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Recombinant adeno-associated viral (rAAV) vectors hold promise for delivering genes for heart diseases, but cardiac-specific expression by the use of rAAV has not been demonstrated. To achieve this goal rAAV vectors were generated expressing marker or potentially therapeutic genes under the control of the cardiac muscle-specific alpha myosin heavy chain (MHC) gene promoter. The rAAV-MHC vectors expressed in primary cardiomyocytes with similar kinetics to rAAV-CMV; however, expression by the rAAV-MHC vectors was restricted to cardiomyocytes. rAAV vectors have low cytotoxicity, and it is demonstrated here that rAAV fails to induce apoptosis in cardiomyocytes compared with a recombinant adenoviral vector. rAAV-MHC or rAAV-CMV vectors were administered to mice to determine the specificity of expression *in vivo*. The rAAV-MHC vectors expressed specifically in cardiomyocytes, whereas the control rAAV-CMV vector expressed in heart, skeletal muscle, and brain. rAAV-MHC transduction resulted in long term (16 weeks) expression of human growth hormone following intracardiac, yet not intramuscular, injection. Finally, we defined the minimal MHC enhancer/promoter sequences required for specific and robust *in vivo* expression in the context of a rAAV vector. For the first time we describe a panel of rAAV vectors capable of long term cardiac specific expression of intracellular and secreted proteins.

Myocardial gene therapy represents a promising approach for the treatment of inherited heart diseases, cardiomyopathies, and congestive heart failure (1). Extensive work has demonstrated that recombinant adenoviral (rAd)[1] vectors can efficiently transduce cardiomyocytes *in vivo* (2, 3) to express genes, including the potassium channel (4), sarcoplasmic calcium ATPase-2A (5, 6), and phospholamban (7). However, rAd-mediated gene transfer is limited by immune responses to viral proteins (8–12), which can cause significant myocardial inflammation (13). Designing a delivery system with low cytotoxicity and cardiac-specific gene expression has been a central goal of cardiac gene therapy.

Derived from a non-pathogenic human parvovirus (14), recombinant adeno-associated viral (rAAV) vectors are an alternative to rAd. Their small size and physical stability are advantageous for *in vivo* use, and transgene expression can persist in a wide range of tissues (15–17). Moreover, there is no evidence of cell damage from inflammation after rAAV administration to the liver, skeletal muscle, brain, and heart (16, 18–20), and direct heart injection can program stable transgene expression in cardiomyocytes *in vivo* (19, 21). rAAV vectors are being recognized as vectors for systemic and local long term delivery of gene therapy for clinical diseases (22, 23), yet their promiscuous tropism may lead to the undesirable expression of therapeutic genes in non-targeted cells. This limitation may be circumvented by the use of tissue-specific promoters. Li *et al.* (24) used the muscle creatine kinase (MCK) promoter to specifically express human γ-sarcoglycan in skeletal muscle using rAAV. In addition, liver-, brain-, cancer-, and rod-specific expression has been accomplished using the tissue-specific albumin, enolase, calcitonin, and rod opsin promoters, respectively (25–28).

Among the isoforms encoded by the multigene myosin heavy chain (MHC) family, only the α- and β-MHC isoforms are expressed in cardiomyocytes (29–31). In late fetal life of mice, α-MHC is expressed in the atria while β-MHC is expressed in the developing ventricles. After birth, α-MHC becomes the predominant isoform expressed in mouse ventricles (29, 30). Cell culture studies have demonstrated three different regions within the proximal α-MHC promoter that regulate cardiomyocyte-specific expression (31–33). First, deletion analysis has demonstrated that α-MHC promoter nucleotide −344 is the 5′-boundary of sequences required for high level expression in cardiomyocytes (32). Second, a 30-bp purine-rich negative regulatory (PNR) element was identified in the first intron, between +66 and +96 bp, that is important for cardiomyocyte-specific expression (33). Finally, within the α-MHC promoter a cardiac-specific enhancer spanning bases −344 to −156 was found to direct high level cardiomyocyte-specific expression with a heterologous promoter (32). These elements were characterized *in vitro*, which may not faithfully model expression patterns *in vivo*. Therefore, in addition to developing a vector for cardiac-specific gene therapy, a goal of this project was to develop the use of somatic gene transfer by rAAV as an alternative to germ-line transgenesis for characterizing long term promoter function *in vivo*.

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[1] The abbreviations used are: rAd, recombinant adenovirus; rAAV, recombinant adeno-associated virus; CMV, cytomegalovirus; MCK, muscle creatine kinase; MHC, myosin heavy chain; PNR, purine-rich negative regulatory element; E, enhancer; P, promoter; SMC, smooth muscle cells; X-gal, 5-bromo-4-chloro-3-iodolyl-β-D-galactopyranoside; hGH, human growth hormone; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling; ELISA, enzyme-linked immunosorbent assay; MLC2v, myosin light chain-2v promoter; Ets, E26-transformation specific.

TABLE I
Oligonucleotide sequences

Primer name	Oligonucleotide sequence
α-MHC −344	5-CCACTAGTCCAGTTTGTTCAACTCACCC-3
α-MHC −156	5-CCAAGCTTCAGGGCCCCAAGGTTTGC-3
α-MHC +19	5-AAGGCGCCATGGTGGCGAATTCGGGTTGGAGAAATCTCTG-3
α-MHC +119	5-AAGGCGCCATGGTGGCGAATTCTGACCGGCCTGAACCCCA-3
min CMV −53	5-CCAAGCTTGGGCGGTAGGCGTGTACG-3
min CMV +75	5-AAGGCGCCATGGTGGCGAATTCCAGCTTCGAGGGGAGGC-3′

Stimulation of tissues with trophic hormones may improve diverse organ-specific processes of aging and atrophy. One such protein, human growth hormone (hGH), is a candidate gene for treatment of dilated cardiomyopathy, because clinical and animal studies have indicated that long term administration of hGH may beneficially impact weakened cardiomyocytes (34–36). In these clinical studies, hGH protein was administered systemically, but local production of therapeutic secretable proteins by rAAV may produce higher concentrations in the target organ with fewer systemic side effects and greater therapeutic benefits. As an initial step to advance this approach, we made a panel of rAAV vectors expressing hGH under the control of a cardiac-specific promoter, and here we report the pharmacokinetic properties of delivering a potentially therapeutic gene to the heart. For these studies we cloned fragments of the α-MHC promoter (−344 to +19), a larger promoter fragment containing the PNR (−344 to +119), or the α-MHC enhancer (−344 to −156) together with a heterologous promoter to control transgene expression. The strength and specificity of these α-MHC gene promoter elements in rAAV were validated *in vivo*, and we demonstrate rAAV-MHC-mediated long term cardiac expression of both marker and therapeutic genes with low cytotoxicity.

EXPERIMENTAL PROCEDURES

Plasmids and Viruses—Using primers listed in Table I, promoter fragments were amplified by PCR from the murine α-MHC gene, kindly provided by Dr. Jeffrey Robbins (GenBank™ accession number U71441, University of Cincinnati, College of Medicine, Cincinnati, OH), and they replaced the CMV enhancer/promoter (−582 to +75) between the *Spe*I and *Not*I sites of pAAV-CMV-lacZ (37). For rAAV-MHC-E vectors the minimal CMV promoter (−53 to +75) was ligated to the α-MHC enhancer (−344 to −156). Each construct was verified by DNA sequencing.

rAAV and rAd Vector Production—rAAV vectors were prepared as described previously (16). Briefly, subconfluent 293 cells were co-transfected with vector plasmid and pLTAAVhelp using calcium phosphate (37). Cells were then infected with adenovirus Ad5dl312 (an E1A-deletion mutant) at a multiplicity of infection of 2, and after 72 h the cells were harvested, lysed by three freeze/thaw cycles, Ad was heat-inactivated, and the rAAV virions were purified by cesium chloride gradients. The gradient fractions containing rAAV were dialyzed against sterile PBS, and stored at −80 °C. Dot blot analysis demonstrated particle titers of $1–2 \times 10^{12}$/ml. The E1A-deleted rAd-lacZ vector was prepared as described (38).

Infection of Tissue Culture Cells—Primary neonatal rat cardiomyocytes were prepared and maintained as described (39). HeLa, 293, C2C12, and smooth muscle cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone) and gentamicin in a humidified atmosphere at 37 °C with 5% CO_2. Approximately 24 h after seeding, vector was added directly to the medium of 1×10^6 cells plated in each well of a six-well plate.

Immunohistochemistry and Apoptosis Assay—Neonatal rat cardiomyocytes grown on coverslips were infected with rAAV. 7 days later, the cells were washed in PBS, fixed in 0.25% glutaraldehyde and 2% formaldehyde, washed with PBS, and probed with an α-tropomyosin antibody (Sigma Chemical Co.). After washing, goat anti-mouse IgG Alexa 594 (Molecular Probes) was applied for 1 h. Nuclear staining for DNA fragmentation was performed by the terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) method (Roche Molecular Biochemicals) according to the manufacturer's protocol. Cells were observed using a Nikon Optiphot-2 light microscope, and images were recorded using a Spot (Diagnostic Instruments, Inc.) digital camera. 100 cardiomyocytes were counted, and the means ± S.E. percentage of TUNEL-positive cells was determined in four independent experiments. For cytoplasmic DNA cleavage assays, DNA preparation and agarose gel electrophoresis were performed essentially as described (39).

Direct Myocardial rAAV Injection—Animal care and surgery were performed according to Harvard Institutional Animal Care and Use Committee guidelines and approval, and mice were housed under conventional conditions. 5- to 7-week old ICR mice (Taconic) were anesthetized by injection of ketamine/xylazine and/or inhalation of methoxyfluran (Metofane, Janssen BmbH) before direct injection of rAAV. Using a 30-gauge needle, 50 μl, containing 5×10^{10} particles of the rAAV-lacZ vectors, was injected into liver or left ventricular wall, through the diaphragm, following laparotomy. Injection with the same dose of vectors into the quadriceps femoris was performed accordingly. In addition, 10 μl, containing 1×10^{10} particles of the rAAV-lacZ vectors were injected slowly into the forebrain. The rAAV-hGH vectors were injected into the left ventricular wall of a second group of adult mice. To inject rAAV-hGH vectors directly into mouse myocardium the respiration of anesthetized mice was controlled using a Dwyer SAR-830 small animal ventilator. Through a thoracotomy incision, the heart was exposed, and under direct visualization injected with 50 μl containing 1×10^{11} particles of rAAV-hGH vectors. Afterward the chest cavity was closed, and the mice were allowed to recover.

X-gal Staining and β-Galactosidase Activity—For detection of β-galactosidase activity, freshly excised tissues were fixed in O.C.T. compound (Sakura), flash-frozen, and 16-mm sections were collected on glass slides. These slides were fixed by using 0.25% glutaraldehyde and 2% formaldehyde, washed with PBS, stained overnight with 5-bromo-4-chloro-3-iodolyl-beta-D-galactopyranoside (X-gal) as described (15). The sections were then washed in PBS and counterstained with Nuclear Fast Red.

Detection of Viral DNA by PCR—Total DNA was extracted from tissues using the Puregene DNA isolation kit (Gentra Systems). PCR was used to amplify a 268-bp fragment of the β-galactosidase gene using sense 5′-TCAATCCGCCGTTTGTTCCC-3′ and antisense 5′-TCCAGATAACTGCCGTCACTCC-3′ primers.

hGH Concentration—Blood samples were taken from the retro-orbital vein of anesthetized mice, and the plasma hGH concentrations were determined by ELISA (Roche Molecular Biochemicals).

Statistical Analysis—All results were expressed as means ± S.E. For multiple treatment groups, a factorial analysis of variance was applied followed by Fisher's least significant difference test. A p value of less than 0.05 was considered significant.

RESULTS

Efficient in Vitro Transduction of Cardiomyocytes by rAAV-MHC—The structures of the recombinant vectors rAAV-MHC-PNR, rAAV-MHC-P, rAAV-MHC-E, and rAAV-CMV, used in this study, are shown in Fig. 1A. For the rAAV-MHC-PNR and rAAV-MHC-P vectors expression of the transgene is controlled by the α-MHC enhancer/promoter sequences (−344 to +119) and (−344 to +19), respectively. In the rAAV-MHC-E vector, expression of the transgene is controlled by the α-MHC enhancer (−344 to −156) coupled to the minimal CMV promoter (40, 41). As a control for non-tissue-restricted expression, a rAAV-CMV vector with the constitutively active CMV promoter/enhancer was used (37). First, to examine the potency and kinetics of gene expression in cardiomyocytes, 1×10^6 cardiomyocytes were infected with 1×10^9 viral particles. β-Galactosidase expression was first detected 1 day after infection, and rapidly increased during days 3–7, peaked at day 10, and

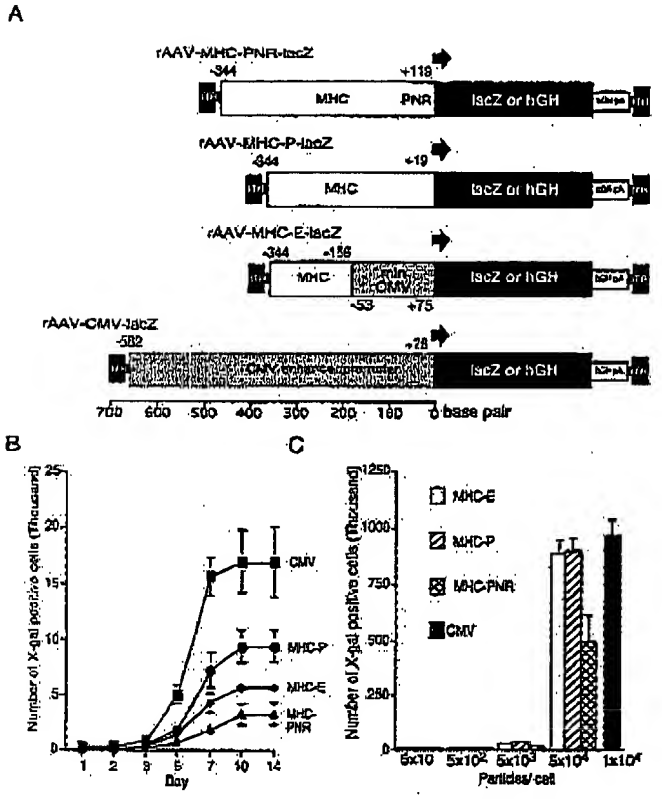

FIG. 1. **The rAAV-MHC vectors transduce cardiomyocytes in a time- and dose-dependent manner.** *A*, each vector contained the β-galactosidase gene (*lacZ*) or human growth hormone (*hGH*) gene, the bovine growth hormone polyadenylation site (*bGH pA*), and the expression cassette was flanked by AAV inverted terminal repeat (*ITR*). The schematics are not drawn to scale: *Clear rectangles* depict the α-MHC promoter fragments; *numbers* indicate the nucleotides for each promoter/enhancer construct. Each fragment used was chosen based on the coordinates from prior published work (29, 33). The minimal CMV promoter (*min CMV*) and CMV enhancer/promoter are shown in *gray*. *B*, the rAAV-MHC vectors transduce cardiac myocytes in a time-dependent manner similar to rAAV-CMV. Starting 1 day after infection of primary rat neonatal cardiomyocytes, the number of X-gal-positive cells were measured. The means ± S.E. from four experiments is shown. *C*, the dose-response relationship of rAAV-MHC vector-transduced cardiomyocytes. Seven days post-infection cardiomyocytes were stained with X-gal, and the number of positive cells were measured. The means ± S.E. from four independent experiments are shown.

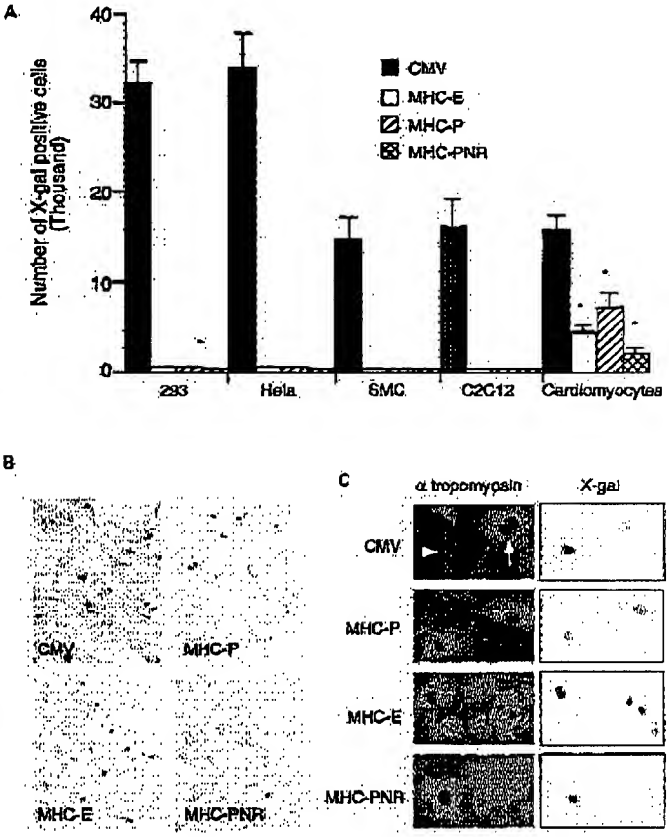

FIG. 2. **rAAV-MHC vectors preferentially transduce cardiomyocytes.** *A*, following infection of 293, HeLa, SMC, C2C12, and cardiomyocytes with 10^3 particles/cell, X-gal staining was performed. The means ± S.E. number of X-gal-positive cells from five independent experiments are presented. *, a statistically significant ($p < 0.05$) difference in number of X-gal-positive cells compared with the number of X-gal-positive 293 infected cells. *B*, neonatal rat cardiomyocytes were stained for β-galactosidase 7 days after infection with 10^4 particles/cell of rAAV vectors. *C*, after infection with 10^4 particles/cell of rAAV vectors for 7 days, cardiomyocytes were immunostained using an α-tropomyosin antibody followed by X-gal staining. Shown are a cardiomyocyte staining positive for both α-tropomyosin and X-gal (*arrow*) and a fibroblast staining just for X-gal (*arrowhead*) following rAAV-CMV transduction.

reached a plateau at day 14 (Fig. 1*B*). To determine the optimal multiplicity of infection 1×10^6 cardiomyocytes were infected with increasing amounts of rAAV, and after 7 days the cells were stained for β-galactosidase activity. The number of β-galactosidase-positive cells infected by the rAAV-MHC vectors demonstrated a dose-dependent effect in the range from 5×10^1 to 5×10^4 particles/cell (Fig. 1*C*). A similar threshold effect was observed using rAAV-CMV increased markedly between the range of 1×10^1 to 1×10^4 particles/cell (data not shown).

Cell Specificity of rAAV-MHC Vectors—We tested the specificity of rAAV-MHC-mediated β-galactosidase expression in 293 (human embryonic kidney cell line), HeLa (human cervical carcinoma cell line), rat aortic smooth muscle cells (SMC) and C2C12 (mouse myoblast cell line). 293 cells and HeLa cells were stained 3 days after infection with 1×10^9 particles, whereas SMC, C2C12 cells, and cardiomyocytes were stained after 7 days, which for each cell type was the peak of their expression. As shown in Fig. 2*A*, rAAV-CMV vector-infected cells showed robust β-galactosidase staining in every cell line, whereas the rAAV-MHC vectors produced few positive cells in the non-cardiomyocyte cell lines (Fig. 2*A*). All three rAAV-MHC vectors produced β-galactosidase in cardiomyocytes (Fig. 2, *A* and *B*). Although rAAV-CMV produced a greater number of positive 293 and HeLa compared with cardiomyocytes, the rAAV-MHC vector produced a far greater number of positive cardiomyocytes than all other cell lines (Fig. 2*A*). Of the three rAAV-MHC vectors, the rAAV-MHC-PNR vector produced the lowest number of β-galactosidase-positive cardiomyocytes (Figs. 1*B*, 2*A*, and 2*B*). Although rAAV-CMV produced similar numbers of β-galactosidase-positive C2C12 cells as cardiomyocytes, rAAV-MHC-E, rAAV-MHC-P, or rAAV-MHC-PNR produced about 47-, 80-, and 93-fold more X-gal-positive cardiomyocytes than C2C12 cells, respectively (Fig. 2*A*). To confirm transgene expression of rAAV-MHC vectors in cardiomyocytes, but not cardiac fibroblasts, which are co-isolated from neonatal rat hearts, we stained infected cells for both β-galactosidase activity and the muscle sarcomeric protein tropomyosin. Expression of β-galactosidase using the rAAV-MHC vectors was mostly restricted to cardiomyocytes (Fig. 2*C*), whereas the rAAV-CMV vector expressed β-galactosidase in both cardiomyocytes and non-myocytes (Fig. 2*C*, *top*). These results demonstrate that the rAAV-MHC vectors express preferentially in cardiomyocytes, and as expected, the PNR element conferred the most cardiomyocyte-specific expression.

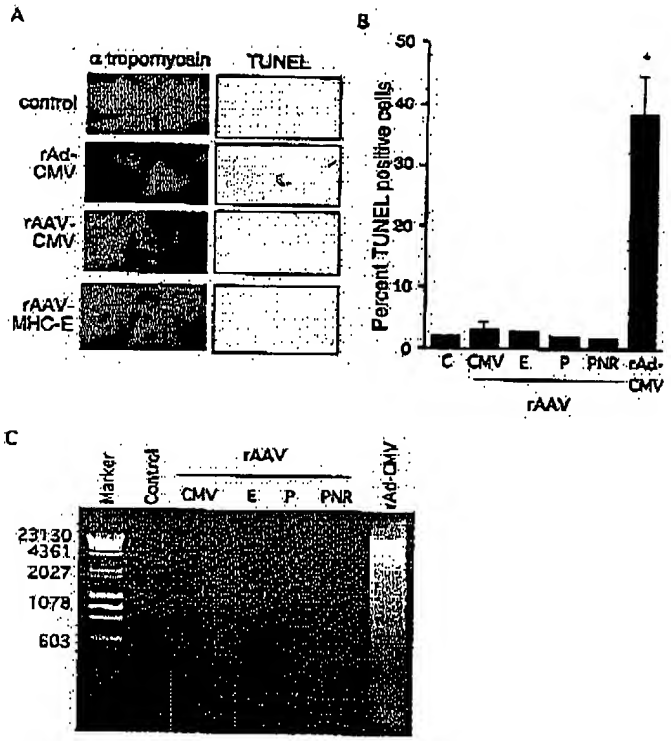

FIG. 3. **rAd but not rAAV vectors induce apoptosis in cardiomyocytes.** *A*, cardiomyocytes were incubated with rAd-lacZ and rAAV-lacZ vectors, then immunostained using an α-tropomyosin antibody followed by TUNEL analysis. Following rAd-CMV infection, a *purple-staining* apoptotic nuclei that co-stains for tropomyosin is indicated. *B*, summary of the rAd and rAAV effects on apoptosis. The percentage of TUNEL-positive cells is presented as the means ± S.E. from three independent experiments. *PNR*, rAAV-MHC-PNR; *P*, rAAV-MHC-P; *E*, rAAV-MHC-E; and *C*, rAAV-CMV. *, significantly more TUNEL-positive cells ($p < 0.05$) compared with uninfected control. *C*, rAd-induced DNA fragmentation in cardiomyocytes. Cytoplasmic DNA isolated from uninfected cardiomyocytes (control) and from rAd- and rAAV-infected cardiomyocytes was subjected to gel electrophoresis alongside molecular weight markers whose sizes are shown on the *left* in base pairs. The gel demonstrates a ladder of DNA bands following rAd transduction.

Transduction of Primary Cardiomyocytes by rAAV Is Non-cytopathic—Recombinant adenoviral vectors can have direct cytotoxic effects whereas rAAV vectors are known to be far less cytopathic. To more closely analyze cytotoxicity profile of the rAAV-MHC vectors, TUNEL analysis was performed to monitor the induction of apoptosis. Infection with 5×10^4 rAAV-CMV vector particles/cell for 8 days and 100 rAd-CMV plaque-forming units/cell for 3 days resulted in every cardiomyocyte nucleus staining positive for β-galactosidase (data not shown). Following infection, rAAV- and rAd-infected cells were subjected to TUNEL analysis at 8 and 3 days, respectively. The rAAV-CMV vector did not increase the number of TUNEL-positive cells compared with the non-infected control cells, whereas incubation with the rAd-CMV vector markedly increased the number of TUNEL-positive cells (Fig. 3, *A* and *B*, $p < 0.005$, compared with control cells) as did treatment of cells with H_2O_2 (data not shown) (39). To confirm the occurrence of apoptosis, we examined DNA fragmentation by agarose gel electrophoresis. Although cytoplasmic DNA extracted from cardiomyocytes after rAd-CMV infection showed prominent ladder formation, incubation with the rAAV-CMV vector did not induce DNA cleavage (Fig. 3*C*). Infection with 5×10^4 rAAV-MHC vector particles/cell also did not induce apoptosis in primary cardiomyocytes (Fig. 3, *B* and *C*). Despite infection with more particles and for a longer time, we found that rAAV are less likely to induce apoptosis in cardiomyocytes.

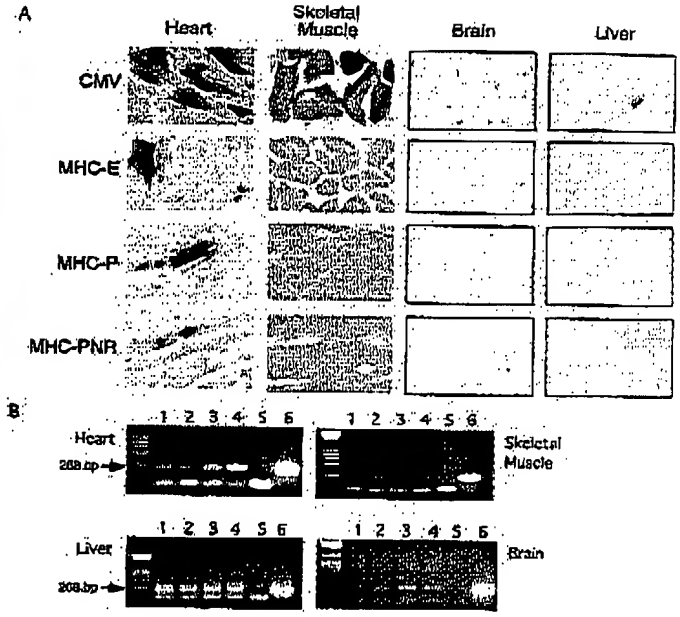

FIG. 4. **The rAAV-MHC vectors preferentially express in heart.** *A*, four mice, 5–7 weeks old, for each construct and each organ, were injected with 5×10^{10} particles of rAAV-lacZ vectors into left ventricular wall, skeletal muscle, and liver, and 1×10^{10} particles of rAAV-lacZ vectors were injected into the brain. After 4 weeks, frozen sections of these tissues were stained for β-galactosidase activity. Representative images demonstrating nuclei with intense blue staining nuclei are shown. *B*, PCR amplification of the rAAV-lacZ genome from injected tissues. Total DNA from each injected tissue was isolated and assayed for the presence of AAV-lacZ sequences by PCR. *Lane 1*, rAAV-CMV; *lane 2*, rAAV-MHC-E; *lane 3*, rAAV-MHC-P; *lane 4*, rAAV-MHC-PNR; *lane 5*, no virus control; *lane 6*, pAAV-CMV-lacZ plasmid. The primers are designed to amplify a 268-bp fragment from the *lacZ* transgene region of the rAAV vectors.

In Vivo Transduction of Adult Mouse Tissues by rAAV-MHC-lacZ Vectors—To determine how efficiently the rAAV-MHC vectors could transduce cardiomyocytes *in vivo*, 5×10^{10} particles of rAAV vectors were injected through the diaphragm into the cardiac wall of 5- to 7-week-old mice. Mice were killed 4 weeks after injection, and assayed for β-galactosidase expression in the myocardium. Positive cells were localized to the injection site. After rAAV-CMV-lacZ administration, β-galactosidase expression was observed in all tissues except liver (only two positive cells), as expected (16), (Fig. 4*A*, *top row*). Each rAAV-MHC vector expressed β-galactosidase in the heart 4 weeks post injection, with the majority of β-galactosidase staining cells being cardiomyocytes (Fig. 4*A*). rAAV vectors were not only injected into the left ventricular wall but also into the liver, quadriceps femoris muscle, and brain. Interestingly, there were very few β-galactosidase-positive cells in both femoral muscle and brain tissues 4 weeks after rAAV-MHC-lacZ injection (Fig. 4*A*), and we did not observe positive cells following liver injection. There was no evidence of myocardial and skeletal muscle inflammation detected by hematoxylin and eosin staining in rAAV-CMV-lacZ-injected hearts (Fig. 4*A*). To confirm gene transfer following injection of the four recombinant viruses, total DNA was isolated from each tissue, and the presence of rAAV sequences was confirmed by PCR. Agarose gel electrophoresis demonstrated the expected 268-bp PCR product in all tissues injected with the rAAV-lacZ vectors (Fig. 4*B*). Thus, rAAV DNA was detected by PCR in all injected tissues, yet the rAAV-MHC-lacZ vectors expressed β-galactosidase significantly only in heart muscle.

Efficient in Vivo Transduction of Adult Mouse Heart by rAAV-MHC-hGH Vectors—To develop potentially therapeutic

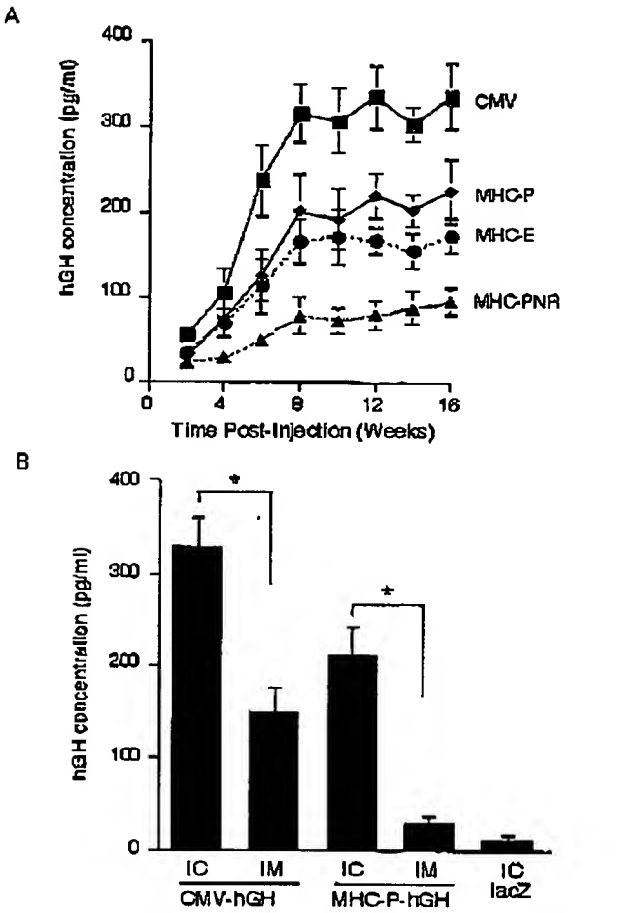

FIG. 5. **Long term expression of rAAV-MHC-hGH vectors.** *A*, following direct injection, the rAAV-MHC-hGH vectors induce long term cardiac expression. 1×10^{11} particles of each rAAV vector were directly injected into the hearts of adult ICR mice ($n = 4$ for each group). Blood was taken from the mice every 2 weeks, and the concentration of hGH in serum was measured by ELISA. The means ± S.E. for each time point are shown. *B*, the rAAV-MHC-P-hGH vector specifically expresses hGH in cardiac muscle. Adult ICR mice ($n = 3$) received intracardiac (*IC*) injections of 1×10^{11} particles or intramuscular (*IM*) injections of 2×10^{11} particles of each rAAV vector, respectively. Twelve weeks after infection, the serum concentration of hGH was measured by ELISA. The means ± S.E. serum hGH concentration are shown. *, a statistically significant ($p < 0.05$) difference in serum hGH concentration following intramuscular and intracardiac injection.

vectors for cardiomyopathy, and to analyze the relative long term expression strength of each rAAV-MHC vector, we exchanged the *lacZ* gene for the hGH gene in the rAAV-MHC vectors (Fig. 1*A*). After direct left ventricular wall injection of 1×10^{11} particles of rAAV, hGH levels rose in a biphasic manner, whereas the hGH signal remained below the limit of detection in serum from control mice injected with an lacZ-expressing rAAV (less than 25 pg/ml). Following rAAV-CMV and rAAV-MHC-P injection, the hGH level became significantly ($p < 0.05$) elevated over lacZ control after 4 weeks. hGH levels increased above baseline at 6 and 8 weeks following administration of rAAV-MHC-E and rAAV-MHC-PNR, respectively (Fig. 5*A*). For each vector, the hGH level continued to rise during the first 8 weeks and remained stable until the end of the experiment after another 8 weeks. The promoter strength based on hGH levels is rAAV-CMV > rAAV-MHC-P > rAAV-MHC-E > rAAV-MHC-PNR. Finally, to further compare the strength and specificity of expression, rAAV-CMV-hGH and rAAV-MHC-P-hGH vectors were either injected directly into heart (IC) or femoral muscle (IM). The serum level of hGH following IM injection of rAAV-MHC-P-hGH was not significantly elevated compared with the lacZ control (means ± S.E. 28.0 ± 12.4 *versus* 11.5 ± 7.5, $p = 0.65$); however, following IC injection, hGH levels were increased 7.5-fold (Fig. 5*B*). By comparison, hGH levels were significantly elevated following both IM and IC injection of rAAV-CMV-hGH. These results demonstrate that rAAV regulated by the α-MHC promoter had long term transgene expression in the heart, similar to the CMV promoter, yet with greater myocardial specificity.

DISCUSSION

In this report, we describe rAAV vectors regulated by elements of the α-MHC promoter, which allow long term transgene expression predominantly in cardiomyocytes. Specific gene expression of rAAV-MHC vectors was shown both in primary neonatal cardiomyocytes *in vitro* and in heart muscle of adult mice *in vivo*. The long term and tissue-specific expression pattern of these vectors presents the potential to develop cardiomyocyte-specific gene therapy as a treatment modality of cardiomyopathy.

The expression kinetics of these constitutive and tissue-specific promoters in the context of rAAV were similar in both primary cultured cardiomyocytes and heart muscle *in vivo*. All four vectors shared a similar profile of expression with low expression soon after infection (<3 days in culture), followed by a steep rise and then a plateau. This pattern probably reflects the dynamics of uptake, viral genome processing, and expression of rAAV that has also been seen in liver (42) and skeletal muscle (43). Differences in promoter strength were reflected in the steepness of their slope and height of their plateau. The *MHC* (−344 to +19) promoter region is perhaps the smallest region of the α-MHC promoter that produces cardiomyocyte-specific expression. This is supported by our finding that the rAAV-MHC-P vector produced the greatest number of β-galactosidase staining cardiomyocytes and human growth hormone level (Figs. 1*B* and 5*A*). In addition, it should be noted that the rAAV-MHC-P-hGH vector was not significantly active in skeletal muscle compared with a control (Fig. 5*B*). We also confirmed earlier studies that identified a cardiomyocyte-specific enhancer from −344 to −156 to be capable of specific high level expression in cardiomyocytes (32). Contained within this region are binding sites for myocyte-specific enhancer-binding factor, GATA, and serum response factor proteins that are important for cardiac-specific gene expression. Our studies show that the rAAV-MHC-E vector is capable of significant and specific expression in primary cardiomyocytes and that the minimal CMV promoter can be used as a heterologous promoter with a cardiac-specific enhancer to target cardiomyocyte-specific expression in the context of rAAV. Hagstrom *et al.* (44) created a hybrid rAAV vector regulated by an α-skeletal muscle actin promoter and CMV enhancer/promoter that produced high level expression in all cells. The minimal CMV promoter has also been coupled to a tetracycline-responsive element and been used in rAAV for regulated transcription (41). It may be possible to use rAAV-MHC to express the tetracycline-activator for cardiac-specific and regulated transcription. The significance of our findings lies in the future use of small enhancer sequences, or minimal promoter elements rather than large promoters, to drive tissue-specific expression by rAAV.

Compared with rAAV-CMV, the rAAV-MHC vectors did not significantly express in HeLa, 293, C2C12, and smooth muscle cells, demonstrating that expression by the rAAV-MHC vectors is specific to cardiomyocytes. We have also confirmed the role of the highly conserved PNR element in the first α-MHC gene intron as being important for cardiomyocyte-specific transcription (33). Although expression by rAAV-MHC-PNR was lower in all cells tested, it was particularly low in non-cardiomyo-

cytes, which contributed to its greater specificity compared with rAAV-MHC-E and rAAV-MHC-P. Consistent with our *in vitro* data (Fig. 2), we found that rAAV-MHC-P and rAAV-MHC-E expressed hGH significantly stronger than rAAV-MHC-PNR *in vivo*, again demonstrating that the PNR negatively regulates transcription (Fig. 5*A*). Within the PNR a palindrome of two high affinity Ets-binding sites has been identified by DNase footprint analysis. PNR binding activity is increased in adult rat hearts subjected to pressure overload hypertrophy, a condition in which α-MHC expression is usually suppressed (33), suggesting that Ets, or an Ets-like factor (45), may be responsible for cardiac-specific α-MHC expression. Using a rAAV-based system, we have extended the *in vitro* results of Gupta *et al.* (33) that the addition of the PNR to the α-MHC promoter reduces promoter activity *in vivo* while improving cardiac specificity. rAAV vectors can therefore be utilized to understand long term promoter function *in vivo*, potentially saving the labor, time, and expense of producing multiple lines of transgenic animals.

Infection with adenovirus can cause cell damage by immune response or direct cytotoxicity (9, 46). Although it has recently been reported that rAAV selectively induces apoptosis in p53-deficient cells, rAAV is non-pathogenic in normal eukaryotic cells (16, 47). For our studies we used the first generation rAd-CMV-lacZ, which expressed strongly in all cultured cardiomyocytes and produced a similar amount of apoptosis as hydrogen peroxide treatment (data not shown) (39). We found that infection of a similar or greater number of rAAV particles did not induce apoptosis compared with the uninfected cells (Fig. 3, *A* and *C*), whereas the first generation rAd produced apoptosis. Even cardiac fibroblasts, which proliferate *in vitro*, did not demonstrate increased apoptosis following rAAV infection (data not shown). In the present study, we compared the cytotoxicity profile of rAAV and first generation rAd, which is known to be cytotoxic. Newer generations of adenoviral vectors demonstrate significantly less cytotoxicity than first generation adenoviral vectors (48, 49). Reduced cytotoxicity and stable maintenance of vector sequence makes rAAV more suitable for long term expression of therapeutic genes and for *in vivo* analysis of promoter function without interference from apoptosis.

rAAV vectors efficiently transduce a variety of cells *in vivo* and are being evaluated for gene therapy of myopathies (15, 17, 19, 20, 24). Transduction of muscle by rAAV results in stable expression (50–52). Using the CMV promoter, rAAV vectors have been used to transfer the human minidystrophin gene to skeletal muscle of mdx mice, resulting in long term correction of their dystrophic degeneration (24). Direct muscle injection of an rAAV vector expressing human γ-sarcoglycan under the control of the MCK promoter induced a significant numbers of muscle fibers expressing γ-sarcoglycan, and improved the histologic pattern of dystrophy in γ-sarcoglycan-deficient mice (53). Previous studies demonstrating efficient viral transduction of muscle have required either direct injection, or at least injection into the arterial supply of a target muscle (19, 21). In agreement with these findings we did not find significant cardiac expression 4 weeks after venous injection of the rAAV-MHC and rAAV-CMV vectors (data not shown). Transduction of skeletal muscle is increased by arterial infusion of rAAV with histamine-induced endothelial permeabilization (54). Future delivery of cardiac gene therapy by venous administration may require improved vector targeting together with endothelial permeabilization (55).

Growth hormone and its local effector insulin-like growth factor-I have been shown to be important for maintaining cardiac mass and performance in adult life, and it has been investigated as a treatment of cardiomyopathy (34–36). We observed long term expression of hGH following myocardial injection for 16 weeks (Fig. 5*A*). Interestingly, injection of the rAAV-CMV vector into the heart produced a greater amount of hGH compared with injection into skeletal muscle (Fig. 5*B*), suggesting that the heart may be more efficient at producing secreted proteins than skeletal muscle or that proteins secreted in the heart have greater access to the circulation. Skeletal muscle can serve as a depot for production of secretable proteins, such as erythropoietin and factor IX (15, 52), and we now demonstrate that cardiac muscle also has this capability. Because long term administration of growth hormone may be a beneficial treatment of cardiomyopathy (34, 35), the potential therapeutic use of these vectors to provide local delivery of hGH in murine cardiomyopathy disease models is now under investigation. Future improvements may depend on alternate cardiac-specific promoters, including the myosin light chain-2v promoter (MLC2v), which is a cardiac-specific promoter active in embryonic and adult ventricular myocardium, including human hearts (56, 57). Franz *et al.* (58) reported that rAd containing the 2100-bp MLC2v promoter expressed at high levels in heart muscle. Although a promoter of this size would limit the transgene size for rAAV, we are nonetheless developing the use of the MLC2v promoter for long term rAAV expression in cardiomyocytes.

In summary, we have demonstrated that the α-MHC promoter and enhancer regions can direct rAAV-mediated cardiomyocyte-specific expression both *in vitro* and *in vivo*. The functional importance of the development of rAAV-MHC vectors is underscored by the finding that they have tissue-specific expression and low cytotoxicity and produce long term transgene expression in this tissue. Taken together, these rAAV-MHC vectors will be useful experimental tools, particularly to validate therapeutic approaches in animal disease models. Finally, we strongly consider that this approach may yield effective cardiomyocyte-specific therapies for human cardiomyopathies such as inherited heart diseases and congestive heart failure.

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Rescue of hereditary form of dilated cardiomyopathy by rAAV-mediated somatic gene therapy: Amelioration of morphological findings, sarcolemmal permeability, cardiac performances, and the prognosis of TO-2 hamsters

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The hereditary form comprises ≈1/5 of patients with dilated cardiomyopathy (DCM) and is a major cause of advanced heart failure. Medical and socioeconomic settings require novel treatments other than cardiac transplantation. TO-2 strain hamsters with congenital DCM show similar clinical and genetic backgrounds to human cases that have defects in the δ-sarcoglycan (δ-SG) gene. To examine the long-term *in vivo* supplement of normal δ-SG gene driven by cytomegalovirus promoter, we analyzed the pathophysiologic effects of the transgene expression in TO-2 hearts by using recombinant adeno-associated virus vector. The transgene preserved sarcolemmal permeability detected *in situ* by mutual exclusivity between cardiomyocytes taking up intravenously administered Evans blue dye and expressing the δ-SG transgene throughout life. The persistent amelioration of sarcolemmal integrity improved wall thickness and the calcification score postmortem. Furthermore, *in vivo* myocardial contractility and hemodynamics, measured by echocardiography and cardiac catheterization, respectively, were normalized, especially in the diastolic performance. Most importantly, the survival period of the TO-2 hamsters was prolonged after the δ-SG gene transduction, and the animals remained active, exceeding the life expectancy of animals without transduction of the responsible gene. These results provide the first evidence that somatic gene therapy is promising for human DCM treatment, if the rAAV vector can be justified for clinical use.

In spite of a steady progress in the pharmaceutical treatment of dilated cardiomyopathy (DCM), the patient's prognosis is still poor (1). Cardiac transplantation is the most life-saving therapy of DCM at the advanced stage, although it includes a wide variety of medical and socioeconomic problems. Another potential strategy including gene therapy is urgently required (2), particularly in the infantile or juvenile cases when it is difficult to repeat cardiac transplantation along their growth. An animal model is useful for developing a new treatment. Cardiomyopathy (CM) hamster is a representative model of human hereditary CM (3) and is divided into hypertrophic CM (BIO 14.6 strain) and DCM-inbred sublines (TO-2 strain), both of which descended from the same ancestor (4). In 1997, two groups independently identified the responsible gene as δ-sarcoglycan (δ-SG) in the strain BIO 14.6 (5, 6). We also have determined the breakpoint of δ-SG gene at the intron 1 in both BIO 14.6 and TO-2 strains (6). In human cases with DCM, the similar δ-SG gene defect has been reported in four families, and one member required heart transplantation (7).

Gene therapy might be promising for the DCM treatment of hereditary origin. Both the limited area and transient duration after the *in vivo* gene transfer has disturbed a functional evaluation of the transfected hearts (8, 9). The *in vivo* transduction of normal δ-SG gene by recombinant adeno-associated virus (rAAV) has made it possible to induce both the transcript and transgene in appreciable amounts and ameliorate cardiac dysfunction up to 10 and 20 weeks (Ref. 10; Fig. 1). This vector has been proven nonpathogenic (11, 12) and has been tried for the therapy of human patients with cystic fibrosis (13) or hemophilia B (14). We hypothesized that supplementation of normal δ-SG before the onset of disease in the DCM animals by a mean of *in vivo* gene transfer may rescue the animals from the development and progression of the disease. Here, we report that an efficient rAAV-mediated δ-SG gene transfer into hearts of TO-2 hamsters resulted in a dramatic rescue of animals from developing the disease, with long-term improvements of morphological lesions, physiological indices at both the cellular and organ levels, and the prognosis.

Materials and Methods

Experimental Animals and Specific Antibodies. Normal (n = 12) and TO-2 strain hamsters (n = 50) with the early onset of DCM (4, 6, 9) were purchased from Bio Breeders (Fitchburg, MA). All of the animals were male and 5 weeks old at the gene transduction, housed under diurnal lighting, and allowed food and tap water *ad libitum*. TO-2 strain hamsters were divided into the following three subgroups: (*i*) totally untreated animals (n = 6); (*ii*) transfected by the reporter gene, Lac Z, alone (n = 24); and (*iii*) cotransfected by Lac Z and δ-SG gene with normal sequence (n = 20). Polyclonal and site-directed antibody to δ-SG was prepared in high titer with synthetic peptide (GPKAVEAY-GKKFEVKT) as a specific epitope of which amino acid sequence was deduced from the cloned cDNA (6). Monoclonal antibody to β-Gal was obtained from NovoCastra, Newcastle, U.K.

Abbreviations: DCM, dilated cardiomyopathy; CM, cardiomyopathy; δ-SG, δ-sarcoglycan; rAAV, recombinant adeno-associated virus; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; LVP, left ventricular pressure.

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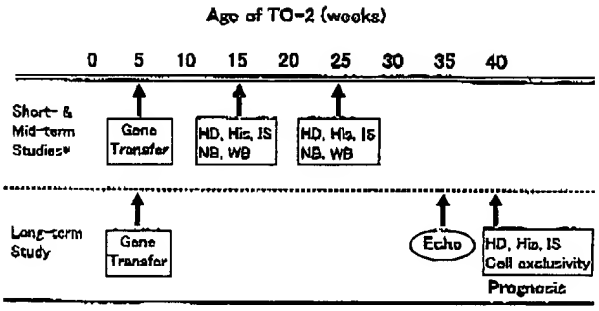

Fig. 1. Protocol for the assessment of gene therapy using rAAV vector. Unlike a previous report (10), the present study was focused mainly on the long-term efficacy and improvement of the animal's prognosis that might be the most important to verify a rationale to develop a novel therapy. HD, hemodynamics; His, histological examinations; IS, immunostaining; NB, Northern blot; and WB, Western blot.

rAAV Vector Construction and Protocol for Gene Delivery *in Vivo*. pW1, an rAAV plasmid containing a reporter gene, Lac Z, flanked by the inverted terminal repeats of AAV genome, pHLP19, a helper plasmid with rep and cap genes, and pladeno-1, harboring adenovirus E2A, and E4 and VA genes were used for the rAAV-reporter gene production (15). pWSG harboring the δ-SG expression cassette driven by the same cytomegalovirus promoter was prepared for rAAV-δ-SG biosynthesis. These rAAVs were produced in 293 cells in culture and purified. The titer of each vector was determined, as described (10).

Under open chest surgery with constant volume ventilation (Model 683, Harvard Bioscience, South Natick, MA; ref. 10), rAAV-reporter gene (Lac Z) alone or the mixture of rAAV-Lac Z and rAAV-δ-SG gene was administered intramurally to the cardiac apex and two sites in the left ventricular free wall (10 μl each: 8.4×10^{10} and 6×10^{10} copies for Lac Z and δ-SG gene in total, respectively). Then, animals were cared for 35 weeks after the transduction in the Infection Research Laboratory under Guidance for Animal Facility: Maintenance and Housing Conditions.

The long-term protocol to follow both physiological and pathological effects *in vivo* and postmortem after the gene transfer is summarized in Fig. 1. Because the present study was addressed mainly to the examination of sarcolemmal integrity and myocardial contractility (both *in vivo*), we analyzed leaky cardiomyocytes secondary to the degradation of transmembrane dystrophin-related proteins (DRP, ref. 9) and wall motion by high-resolution echocardiography, in addition to the hemodynamic studies and prognosis analysis.

Evaluation of Pathological Alterations. The transgene expression was not restricted to the injected sites; a distant region also was transfected (10). Accordingly, wall thickness was measured at the four portions; interventricular septum, left ventricular free wall opposite to the septum, anterior and posterior free walls of the left ventricular cavity in the cross section after staining by hematoxylin and eosin and then summed. For the semiquantification of tissue calcification, we scored as follows, depending on the degree: 0 points, without calcified region; 1 point, with one calcified spot; 2 points, with two regions; and 3 points, with more than three regions and/or huge, elongated, or fused region. The score was summed in four cross sections between the apex and mitral annulus without notifying the observer as to which site and which vector was transfected.

Morphological Analyses and Evaluation of Sarcolemmal Permeability. For the immunostaining of the reporter transgene (β-Gal) and δ-SG, we used adjacent serial sections with a specific antibody to each protein because of the increased sensitivity and specificity (16). To evaluate the rAAV-δ-SG treatment, sarcolemmal integrity was analyzed by i.v. injection of 1% (vol/vol) Evans blue dye, which was kept circulating for 3 h before killing the animals at 35 weeks after the gene transduction. The dye was excluded cardiomyocytes that preserves normal sarcolemmal permeability but is taken up by the cardiomyopathic cells with leaky cell membrane (17, 18). The immunostaining of δ-SG by FITC-labeled second antibody to rabbit IgG (NovoCastra, Newcastle, U.K.) and Evans blue were visualized under double fluorescence microscopy with a Nikon F800 equipped with a green activation filter (546 nm with 12 nm band-pass) for the excitation and barrier filter (590 nm) for the emission. The site where β-Gal, a transgene of reporter, was identified by immunostaining (Fig. 2), recorded by digital camera at 200× magnification, and used for the analysis of other transgene expression or Evans blue uptake. The areas of positive cytoplasm for Evans blue or δ-SG were measured by planimetry (10) for the assay of membrane permeability and efficacy of the transduction, respectively.

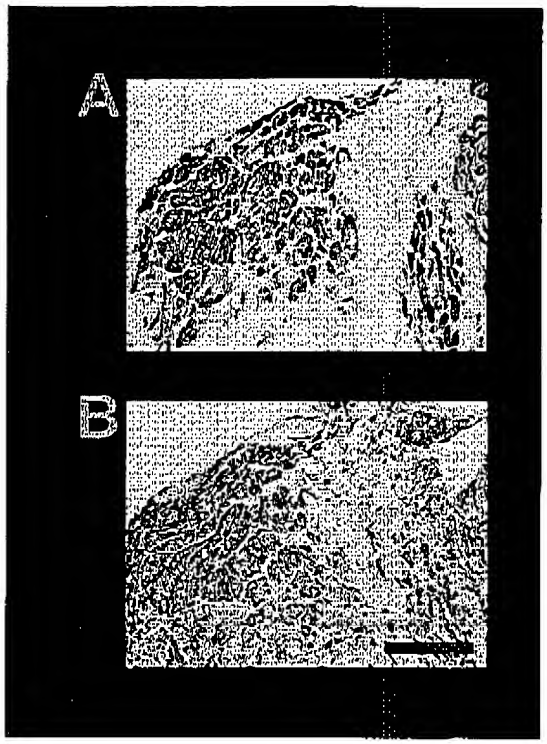

Fig. 2. Efficient expression of reporter β-Gal (*A*) or δ-SG (*B*) in serial sections after the rAAV-mediated gene transduction to the TO-2 hamster hearts that had lacked δ-SG gene (6, 9). Original magnification, ×4. (Bar = 100 μm.)

***In Vivo* Assessment of Cardiac Contractility and Hemodynamics.** Mechanical performances were determined by several observers who were not aware of the administered vectors and the injection site. Before both echocardiographic and hemodynamic measurements, the concentration of the gas anesthetic isoflurane was reduced to 1% and maintained for 20 min to stabilize the hemodynamics (19). Both the left ventricular systolic dimension (LVDs) and left ventricular diastolic dimension (LVDd) were determined by high-frequency (13 MHz) echocardiography (EUB 6000, Hitachi, Tokyo) under visualizing short axis of the left ventricle at 30 weeks after the gene transfer. Because these dimensions were still too inaccurate to determine the actual diameter of the left ventricular cavity even using the two-

dimensional view, we measured the percent fractional shortening (FS) and calculated the ejection fraction (EF) by Teicholz's formula.

Thirty-five weeks after the transduction, hamsters were anesthetized again, as described above (19). A catheter-tip transducer (SPR-671, Millar Instruments, Houston, TX) was inserted into the left ventricle through the right carotid artery to measure the left ventricular pressure (LVP), the left ventricular end-diastolic pressure (LVEDP) and the derivative of LVP (dP/dt). For the determination of the central venous pressure (CVP), a heparin-saline-filled polyethylene catheter connected to a pressure transducer was introduced into the superior vena cava through the right jugular vein. The hemodynamic parameters were recorded after A/D transduction on a Power Lab system (A. D. Instruments, Castle Hill, NSW, Australia) at a 1-kHz sampling rate (10, 19).

Evaluation of the Prognosis After Gene Therapy. The final effect of the gene therapy was evaluated on the life-saving action in the TO-2 animals ($n = 20$) with cotransduction of δ-SG plus reporter genes, comparing them with another animal group transfected by reporter gene alone ($n = 24$). All animals were operated on at age 5 weeks, randomly allocated for each treatment, and housed for 40 weeks, which exceeded the mean lifespan of TO-2 strain hamsters (4). The survival rate was evaluated by Kaplan-Meier analysis.

Statistical Analysis. Preliminary study has revealed that the morphological and physiologic effects of each gene were independent and did not show any additive or synergistic action (10). All values were expressed by the means ± SE and analyzed by paired Student's t test and ANOVA. A P value of less than 0.05 was considered significant.

Results

Wall Thickness, Calcified Lesion, and Transgene Expression. Wall thickness measured at four points of the left ventricular wall of TO-2 heart and summed revealed that the *in vivo* cotransduction of reporter and δ-SG genes ($n = 16$) increased the thickness from 4.4 ± 0.2 to 5.1 ± 0.3 mm ($P < 0.05$), compared with the heart transfected by the reporter gene alone ($n = 10$). These results confirmed previous data that cell width in the myocardium transfected by δ-SG gene was normalized, in part, by the present therapy (10).

The calcified lesion was homogeneously distributed throughout the ventricular wall at random. We semiquantitatively scored the lesion in each animal and summed. TO-2 hearts transfected by the Lac Z gene alone ($n = 10$) showed a 1.46-fold larger score than those treated by both Lac Z and δ-SG genes ($n = 16$, 9.9 ± 0.9 vs. 6.8 ± 1.0, $P < 0.05$). These results denote the physiological effect of δ-SG on the progression of calcification.

To identify the transgene of the reporter, immunostaining of β-Gal protein by specific antibody was more sensitive than the classic, histochemical reaction and did not disturb the immunodetection of SG protein secondary to blue color presentation of the reaction product after histochemistry (16). The β-Gal expression was observed at 35 weeks after the transduction (i.e., at age 40 weeks) in cardiac muscle of TO-2 hamsters. Combined with a previous report that the transgene also was documented at 10 and 20 weeks after the gene transfer (10), the present results indicate that the transgene expression with rAAV continued throughout the animal's life (4).

The transgenes of both reporter and δ-SG were clearly detected in the same part of serial sections (Fig. 2). The β-Gal was shown exclusively in the cytoplasm of cardiomyocytes (Fig. 2*A*), indicating that β-Gal did not require translocation after the biosynthesis. It should be noted that most myocardial cells presenting β-Gal matched those cells exhibiting δ-SG (Fig. 2*B*). In contrast, the expression of δ-SG was not restricted to sarcolemma, and cytoplasm in some cardiomyocytes also was stained, similar to skeletal muscle (17, 18). Furthermore, it might be quite meaningful to detect the transgene expression in ventricular working muscle cells, His-Purkinje bundle, and coronary smooth-muscle cells (Fig. 2*B*); ventricular myocytes look to be more preferentially transfected than the conduction system.

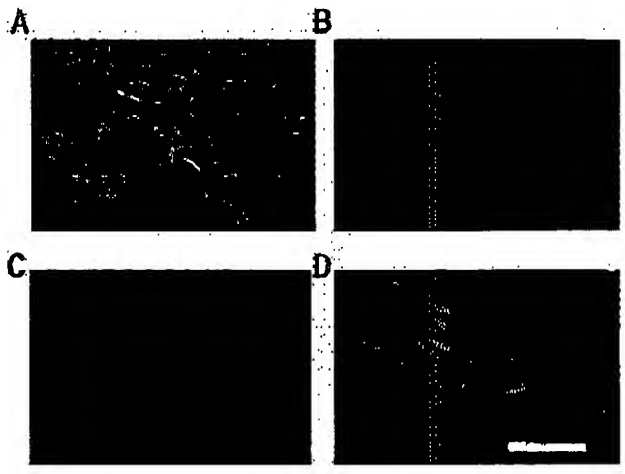

Fig. 3. Mutual cell-exclusivity of δ-SG expression and Evans blue uptake. After the cotransduction of reporter gene plus normal δ-SG gene (*A* and *B*) or transduction of the reporter gene alone (*C* and *D*) for 35 weeks to TO-2 hamster hearts, the transgene of δ-SG and the cells with leaky sarcolemma were detected by double fluorescence with FITC-labeled antibody (*A* and *C*) and Evans blue (*B* and *D*), respectively. Original magnification, ×200. (Bar = 100 μm.)

Amelioration of Sarcolemmal Permeability After the Gene Therapy. To examine the nonspecific effect of gene transfer on myocardium, the age-matched TO-2 hamsters transfected by reporter gene alone were used as controls of TO-2 hamsters cotransfected by reporter gene plus δ-SG gene. At 35 weeks after the transduction, cardiac tissue was collected and examined for δ-SG expression and Evans blue dye uptake by double fluorescence visualization. As expected, cardiac muscle from TO-2 hamsters treated by the reporter plus δ-SG genes revealed the site-specific expression of δ-SG transgene, where the reporter was detected in a serial section. The δ-SG (green fluorescence, Fig. 3*A*) was stained across cardiomyocytes that did not take up Evans blue (red fluorescence, Fig. 3*B*), distinctly showing the mutual cell-exclusivity of δ-SG expression and the dye uptake. In contrast, the TO-2 heart transfected by the reporter gene alone revealed the absence of δ-SG (Fig. 3*C*) and the extensive dye uptake (Fig. 3*D*). The control F1B heart demonstrated no uptake of Evans blue but clear immunostaining of δ-SG (data not shown).

It should be intensified that the rAAV-δ-SG treatment of TO-2 muscle achieved the protection of cardiomyocytes from sarcolemmal leakage as late as 40 weeks old, when some TO-2 hamsters died of heart failure (4). In the distant myocardium where β-Gal or δ-SG was not detected, Evans blue was strongly stained (data not shown). These results unequivocally demonstrate the physiological significance of δ-SG to protect cardiomyocytes from the sarcolemmal deterioration, similar to gene therapy for 10- and 20-week-old hamsters (10).

Quantitative assessment of the relationship between δ-SG staining and Evans blue uptake required counting every positive cell in composite photographs. These data demonstrate that Evans blue-positivity conferred a 5.88-fold protective effect of the gene therapy (20 specimens of 5 animals in each group), because the ratio decreased from 4.48 ± 0.27 to 0.83 ± 0.13 ($P < 0.01$).

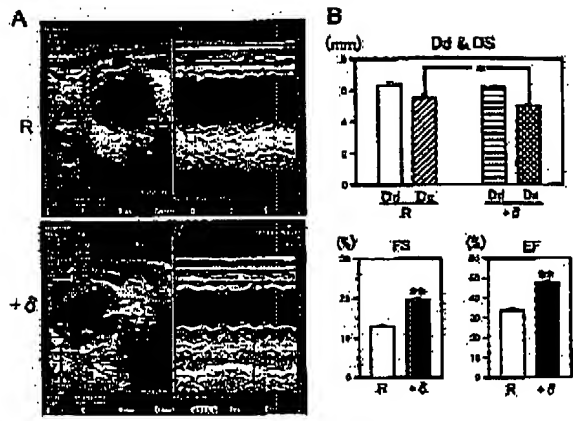

Fig. 4. (*A*) Short-axis view of the left ventricular cavity by high-frequency (13 MHz) echocardiography and M mode recording in TO-2 hamsters. The LVDs and LVDd were determined at 30 weeks after the transduction of reporter gene (R) alone or the cotransduction of R plus normal δ-SG gene (+δ) to TO-2 heart *in vivo*. (*B*) Summary of the LVDd (Dd), LVDs (Ds), fractional shortening (FS), and ejection fraction (EF) in a group transfected by the reporter gene alone (white bar, $n = 10$) or another group cotransfected by the reporter gene plus normal δ-SG genes (black bar, $n = 10$).

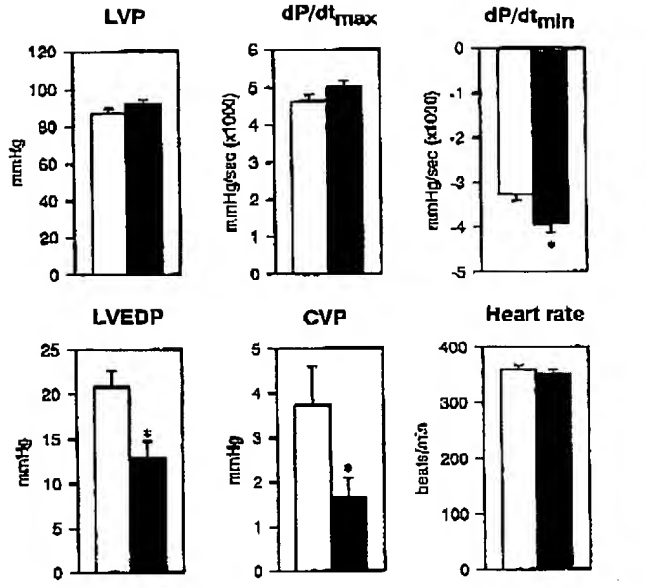

Fig. 5. Comparison of hemodynamic indices at 35 weeks after the gene transduction *in vivo*. LVP, CVP, and the heart rate were recorded under stable anesthesia (10). The LVP was digitized to calculate the maximum derivative (dP/dt_{max}) and the minimum derivative (dP/dt_{min}). White and black bars denote TO-2 hamsters transfected by the reporter gene alone (R) and cotransfected by the reporter plus δ-SG genes (+δ) for 30 weeks, respectively. *, statistical significance between the two groups ($P < 0.05$).

Improvement of Myocardial Contractility and Hemodynamic Indices. High-frequency (13 MHz) echocardiography and its digital recording have made it possible to exactly evaluate the mechanical performances *in vivo* (Fig. 4). Operation procedure at 30 weeks before (Fig. 1) did not disturb visualization of the ventricular cavity (Fig. 4*A*). The *in vivo* cotransduction of reporter gene plus δ-SG gene ($n = 10$) to the TO-2 strain reduced the enlarged LVDs from 5.52 ± 0.18 to 4.98 ± 0.09 mm ($P < 0.05$), compared with the animals ($n = 10$) transfected by the reporter gene alone (Fig. 4*B*). In contrast, the LVDd did not change even after the gene therapy in both groups (6.33 ± 0.18, vs. 6.18 ± 0.11 mm). These results were reflected in the improvement of both percent fractional shortening (FS, 12.9 ± 0.5 vs. 19.5 ± 0.7, $P < 0.01$) and the left ventricular ejection fraction after the transfer of δ-SG gene (LVEF, 33.7 ± 1.2 vs. 47.4 ± 1.3; $P < 0.01$; Fig. 4*B*).

Open chest surgery for the gene transduction did not hamper the exact measurement of the hemodynamics at 35 weeks after the gene transduction (Fig. 5). Cotransduction of both the reporter and δ-SG genes ($n = 18$) distinctly improved the dP/dt_{min} (−3,269 ± 147 vs. −3,955 ± 183 mmHg/sec, $P < 0.05$), the LVEDP (20.8 ± 1.8 vs. 12.8 ± 1.9 mmHg, $P < 0.05$) and the CVP (3.72 ± 0.88 to 1.66 ± 0.43 mmHg, $P < 0.05$), compared with transduction of the reporter gene alone ($n = 12$). Gene therapy did not modify the LVP (86.8 ± 2.6 vs. 91.7 ± 2.4 mmHg), the dP/dt_{max} (4,629 ± 186 vs. 5,000 ± 162 mmHg/sec), or the HR (358 ± 8 vs. 350 ± 8 beats per min).

Prolongation of Life Expectancy After the Gene Therapy. At 5 weeks old, one group of TO-2 hamsters ($n = 20$) was administered reporter gene alone *in vivo* and another group ($n = 24$) was cotransfected by the reporter and δ-SG genes. All animals survived the open chest surgery, indicating that the operational procedure did not cause serious effect on their mortality or morbidity.

The group treated by reporter gene alone started to die at 34 days old and the number of deceased animals gradually increased from 171 to 228 days after the gene transfer (Fig. 6). The death timing supports the previous data in the same strain without gene manipulation (4). In contrast, all animals in another group cotransfected by the reporter plus δ-SG genes survived and remained active. We conclude that the present gene therapy prolonged the survival rate ($P < 0.01$), when the gene responsible for DCM was supplemented *in vivo*.

Discussion

The present study demonstrated that, upon rAAV-mediated efficient δ-SG gene transfer into the heart, TO-2 hamsters can be rescued from developing DCM and survive for at least 40 weeks, which exceeded the lifespan of TO-2 heart without responsible gene transduction, thus drastically improving the disease prognosis.

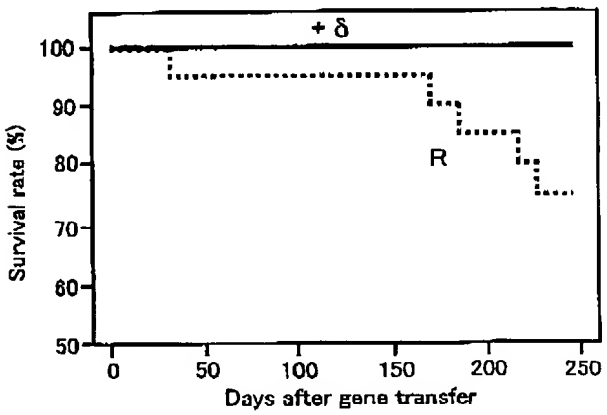

Fig. 6. Kaplan–Meier analysis of TO-2 strain hamsters' survival rate after the transduction of the reporter gene alone (R, $n = 20$) or cotransduction of the reporter plus normal δ-SG genes (+δ, $n = 24$) *in vivo*. Note that the animal group treated by R alone completely matched the prognosis of the animals without the gene manipulation (4), whereas another group treated by +δ survived the operational procedures for the *in vivo* gene transfer and remained active.

DRP links intracellular contractile machinery with extracellular matrix (20, 21). Gene defect and the corresponding protein disruption in the complex commonly induce muscle degeneration with or without cardiac symptoms. In fact, gene mutation of cardiac F-actin, dystrophin, each SG and laminin-α2 in addition to lamin A/C causes DCM in human cases as the chief symptom or a partial sign (20–23). Furthermore, acquired case in rat with myocarditis after enterovirus infection shows DCM-like symptoms secondary to the selective cleavage of dystrophin by protease 2A translated from the virus genome (24). We also have demonstrated that over-administration of isoproterenol to rats caused the selective cleavage of dystrophin, its translocation from sarcolemma to myoplasm, cardiomyocyte apoptosis, and finally acute or subacute heart failure (24). Accordingly, the interruption between intracellular F-actin and extracellular laminin-α2 would fail to preserve the integrity of sarcolemma, resulting in a DCM-like syndrome, irrespective of the hereditary or acquired origin.

Wall thickness was normalized after the gene therapy, and this fact might be a result of improved cell diameter (10) and/or the reduction of calcified lesions in the transfected site. Furthermore, the reduced calcification suggests that the pathogenesis is intrinsic to the deletion of the δ-SG gene *per se* and is not a result of additional deletions other than the δ-SG gene. Although the rAAV type 2 vector is potent for the widespread and long-lasting gene transduction *in vivo*, efficacy was still lower than the case after open chest surgery and confirms our previous results of the gene therapy for short or mid-term period (10). Other methods of gene transfer *in vivo* using intracoronary administration and/or electroporation did not exceed the present level of intramural administration (T.K., M.N., J.N., C.H., and T.T.-o., unpublished data). More efficient gene transfer through coronary circulation, as succeeded in heterotopically transplanted heart after the isolated perfusion (17), would completely restore these pathological alterations before the progression to irreversible degeneration. The apparent discrepancy between the reduced LVDs by echocardiography, and no effect on the LVP or dP/dt_{max} by hemodynamic study, might be explained by the insufficient gene delivery to cover whole heart. Escape of both atria from the gene transduction and their reduced contractility may decrease the preload of both ventricles and would not be reflected to the increment of LVP or dP/dt_{max}. We have reported that transduction of 30–40% cells and 20% protein amount were sufficient for improving hemodynamics, as was verified by immunohistology and Western blotting, respectively, for the level of δ-SG to rescue the animals (10). These results suggest the redundant expression of δ-SG in normal animals.

Based on the results of cardiomyocyte degradation in transgenic mice and their improvement by the pharmaceutical agent with coronary dilating action, K. P. Campbell and coworkers (25, 26) have presented the scheme that loss of δ- or β-SG but not α-SG would cause DCM secondary to the coronary spasm. These results are quite informative for the development of DCM, but may require further studies on the exact pathogenesis because of the following three reasons. (*i*) The same δ-SG gene deletion causes different phenotypes in hamsters; hypertrophic CM at the initial onset followed by DCM in BIO 14.6 strain (4, 6) and DCM as the first symptom in TO-2 strain (4, 6, 9, 10). It might be attractive to assume that an additional mutation in the TO-2 strain causes DCM, overcoming the compensatory hypertrophy that occurs in BIO 14.6 strain (*ii*). After the gene therapy, δ-SG was expressed in not only cardiomyocytes but also in smooth-muscle cells in the coronary artery (Fig. 2*B*). We found no significant difference in the coronary artery caliber between the δ-SG transfected and nontransfected arteries (data not shown). Measurements of local coronary flow with the vasospasm-inducing agents (e.g., acetylcholine or ergonovine) are mandatory to determine the contribution of coronary spasm (27). (*iii*) In addition, another kind of Ca^{2+} entry blocker, nifedipine, with more potent antispastic and more specific coronary dilating action (28) did not improve but rather aggravated the prognosis of this hamster (29).

Cardiac muscle is destined to repeat contraction and relaxation throughout the lifespan, and sarcolemma is supposed to be much more resistant to the expansion-shrinking cycle in the heart than in the skeletal muscle. The lack of a component in DRP is not lethal (20, 21), but its full set may be needed to keep both the membrane integrity and normal lifespan. Actually, present results demonstrate that the exogenously applied Evans blue dye permeated plasma membrane of cardiomyocytes that did not possess δ-SG (Fig. 3 *C* and *D*) when TO-2 hamsters started to die of heart failure (Fig. 6). In contrast, myocardial cells expressing δ-SG after the gene transduction did not take up the dye at the same age (Fig. 3 *A* and *B*). Continuous but gradual leakage of sarcolemma to Ca^{2+} in addition to the Ca^{2+} entry during slow inward current would elevate the intracellular Ca^{2+} level (30) because of the depletion of high-energy phosphates (31) and would activate the endogenous protease, calpain (Ca^{2+}-activated neutral protease, CANP; ref. 32). After that, α−, β− and γ-SG might be hydrolyzed at the posttranslation level (6, 9), because mRNAs for α−, β− and γ-SG were completely preserved (6), and because most of the cytoskeletal proteins, including SGs, are degraded (32) by isolated calpain (M. Koshimizu, H. Yoshida, and S. Takeo, personal communication). On the precise mechanism of DCM progression or coronary spasm, more detailed study would be required on sarcolemmal fluidity (31), humoral factors, or cytokines, including tumor necrosis factor α and/or endothelin (33, 34).

Recent data for some mice with lysosomal storage disease showed hepatocellular carcinoma or angiosarcoma were detected after intrauterine and i.v. administration of rAAV (35). In addition, we found a trace staining of β-Gal protein in liver, spleen, and kidney, suggesting extracardiac transduction of the reporter gene (S.N., T.K., and T.T.-o., unpublished data). Although species other than the transgenic animals described above did not demonstrate any tumor formation (35), and because the heart is one of the privileged organs in relation to tumorigenesis, further study is necessary to test the safety in primates. Present results distinctly indicate that somatic gene therapy with potent vector is promising for human DCM treatment, if the rAAV vector is available for clinical use.

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